Immune infiltration and a ferroptosis-related gene signature for predicting the prognosis of patients with cholangiocarcinoma

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Research Article

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Abstract

Background

Cholangiocarcinoma (CHOL) is a digestive tract tumor with high malignancy and poor prognosis and is extremely challenging to treat. At present, induced cell death holds great promise in tumor therapy. Ferroptosis is a recently proposed pattern of programmed cell death, and numerous studies have shown that it is intimately involved in tumors. However, the roles of differentially expressed ferroptosis-related genes (DEFRGs) in CHOL have not been investigated.

Methods

Our study was based on the The Cancer Genome Atlas (TCGA) database, DEFRGs were obtained to construct a prognostic riskScore model of CHOL by univariate and multivariate Cox regression analyses. Subsequently, the model was evaluated by nomogram construction, survival analysis, receiver operating characteristic (ROC) analysis and exploration of the immune microenvironment, and the mRNA and protein expression levels of each gene in the model were validated by Gene Expression Omnibus (GEO) database and quantitative real-time PCR (qRT-PCR).

Results

We screened four DEFRGs from the TCGA database to construct a prognostic model. The construction of a nomogram confirmed the predictive value of the model for overall survival (OS), and it was confirmed to have high diagnostic value by ROC analysis. The GSEA results suggested that these genes were mainly enriched in ferroptosis- and metabolism-related pathways. Finally, our experimental results validated the expression levels of the four DEFRGs, which were almost consistent with our bioinformatics results.

Conclusion

Our study found that the prognostic model showed extremely high diagnostic and prognostic value and could predict the possibility of immunotherapy, thus providing a new direction for individualized treatment of patients with CHOL.

Introduction

Cholangiocarcinoma (CHOL) is a highly malignant solid tumor originating from the epithelial cells of the biliary tree [1]. As the second most common primary liver tumor, there is currently an increasing trend in morbidity and mortality, with significant regional variation in incidence, accounting for 80% in Asia and South America [2,3]. CHOL has an insidious early onset, and the late clinical manifestations are mostly nonspecific, mainly related to biliary obstruction caused by the tumor. Most patients are often diagnosed

in the middle or advanced stage or when the disease is accompanied by distant metastasis. Although there are many treatment options, the overall prognosis is still poor [4,5]. In addition, current multigenic prognostic models have been widely used to assess the risk of tumors, but a well-established prognostic biomarker for CHOL is still lacking, which makes it difficult for hepatobiliary surgeons to risk stratify CHOL, affecting the choice of treatment regimen. Therefore, we used bioinformatics approaches to mine The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) database and developed new prognostic models, which are essential to improve the prognosis of patients.

Ferroptosis is an iron-dependent regulatory cell death process driven by lipid peroxidation that is different from apoptosis and necrosis, and its characteristic morphological features are mainly characterized by plasma membrane blistering, mitochondrial cristae reduction or disappearance, mitochondrial shrinkage and mitochondrial membrane condensation [6, 7]. Since the concept of ferroptosis was first proposed in 2012, many studies have shown that ferroptosis is strongly correlated with systemic lupus erythematosus, inflammatory bowel disease and tumors [8, 9]. Among them, iron death has attracted extensive attention as a potential treatment modality for tumors, and we can find a large number of studies on ferroptosis in relation to tumors such as hepatocellular carcinoma [10], gastric cancer [11] and lung adenocarcinoma [12]. Currently, some ferroptosis inducers composed of small molecules or nanomaterials have been developed, which not only help to overcome drug resistance but also prevent the metastasis of tumors. However, the roles of differentially expressed ferroptosis-related genes (DEFRGs) in CHOL have not been deeply studied. Therefore, it is urgent for us to further explore them.

In our study, we thoroughly explored the roles of DEFRGs in CHOL and developed a novel prognostic model based on DEFRGs. First, RNA-sequencing (RNA-seq) data and relevant clinical data were extracted from the TCGA database. Next, the FRGs were extracted from FerrDb, the two databases were integrated, and a prognostic model of CHOL composed of four genes was constructed and subsequently validated in the GEO database (GSE26566). Finally, we further elucidated the roles of FRGs in CHOL by quantitative real-time PCR (qRT-PCR) of clinical samples. Studies have shown that this model holds great promise in evaluating CHOL prognosis and guiding immunotherapy. The flow chart of our research is shown in Fig. 1.

Materials And Methods

Data collection of public databases:

In our study, the RNA-seq datasets and corresponding clinical information of 36 CHOL samples and 9 normal samples were derived from the TCGA database (<https://portal.gdc.cancer.gov/repository/>), the data cutoff was October 10, 2021, and differentially expressed genes between tumor and normal samples were selected based on gene attributes by the "limma" package. The cutoff value was set at $|\log_2\text{fold change}| > 1$, and a P value < 0.05 was considered significant. The GSE26566 dataset was acquired from the GEO (<http://www.ncbi.nlm.nih.gov/geo>) database as a validation set, derived from the GPL6104 platform, this dataset includes 104 freshly-frozen tumors and 59 matched non-cancerous livers from

Australia, Europe and the United States, and the experiment performed by Copenhagen University. In addition, we extracted FRGs from the FerrDb (<http://www.zhounan.org/ferrdb/>) database, which is a database containing ferroptosis-related regulators and ferroptosis-related diseases, and then intersected the data with the above FRGs to obtain DEFGRs.

Establishment and validation of DEFGRs prognostic model:

First, univariate Cox regression analysis was applied to identify the DEFGRs with potential prognostic value, a prognostic model was constructed, and the correlation coefficient was obtained by multivariate Cox regression. Then, the riskScore of CHOL patients was determined by the following formula: $\text{RiskScore} = e^{(\beta_1 \times \text{expression of STMN1}) + (\beta_2 \times \text{expression of EGFR}) + (\beta_3 \times \text{expression of ALOX5}) + (\beta_4 \times \text{expression of BNIP3}) - 0.09}$ (β values for the correlation coefficient and a constant value of 0.09). Then, according to the median value of the riskScore, patients were divided into high- and low-risk groups for further analysis.

Construction of nomogram:

Univariate and multivariate Cox regression analyses were used to explore whether the riskScore and clinical parameters could be used as independent prognostic factors for CHOL patients. Subsequently, the specificity and sensitivity of the prognostic model were verified by receiver operating characteristic (ROC) curve analysis. In addition, based on clinicopathological parameters and riskScore, we constructed a nomogram by using the "regplot" R package to evaluate the 1-, 3-, and 5-year overall survival of CHOL patients.

Bioinformatics analysis:

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the above DEFGRs were performed using the "clusterprofiler" package and then visualized using "ggplot". Then, we used the single-sample gene set enrichment analysis (ssGSEA) function from the "gsva" package in the R language to calculate individual enrichment scores for each paired sample and gene set [13, 14], allowing quantification of the extent of immune cell infiltration in tumors. The 24 immune cells involved in this study were referenced to a previous study by our group [15]. Furthermore, we explored immune-related pathways involved in prognostic models.

Patients:

We randomly collected 10 pairs of fresh CHOL specimens and adjacent normal tissue specimens from the First Affiliated Hospital of Chongqing Medical University from July to November 2021. This study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, and all patients signed informed consent forms, and all experiments were performed in accordance with relevant guidelines and regulations.

Quantitative real time PCR (qRT-PCR):

Total RNA was extracted from 10 pairs of fresh CHOL and paracancerous tissues with TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China). Total RNA was reverse transcribed into cDNA using a

PrimeScript™ RT kit (Takara Biotechnology Co., Ltd.). Then, cDNA was amplified with specific primers, and the primer sequences involved in this study are shown in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of genes of interest using β -actin as a standard.

Table 1
Sequences of the primers utilized for quantitative real-time PCR

Name	Sequence
STMN1	
Forward (5'-3')	5'-GTACTTCTGGACTCACGGGC-3'
Reverse (5'-3')	5'-AAGGCAAGAGTGGTCTGCTC-3'
ALOX5	
Forward (5'-3')	5'-AAAGAACTGGAAACACGTCAGAAA-3'
Reverse (5'-3')	5'-AACTGGTGTGTACAGGGGTCAGTT-3'
BNIP3	
Forward (5'-3')	5'-AACTCAGATTGGATATGGGATTGG-3'
Reverse (5'-3')	5'-AGAGCAGCAGAGATGGAAGG-3'
EGFR	
Forward (5'-3')	5'-TCTCAGCAACATGTCGATGG-3'
Reverse (5'-3')	5'-TCGCACTTCTTACACTTGCG-3'

Statistical analysis:

In our study, the statistical analysis was completed with R 4.1.5, SPSS (version 25) and GraphPad Prism software (version 8.0). Student's t test was performed to analyze the difference in gene expression between the two groups of continuous variables, and Pearson's correlation analysis was used. Univariate and multivariate Cox regressions were used to analyze the hazard ratio (HR) and 95% confidence interval (CI) and assess the prognostic value. Generally, a P value < 0.05 was considered statistically significant.

Results

Screening of DEFRGs in CHOL patients:

The RNA expression data and corresponding clinical information of 36 CHOL and 9 adjacent normal samples were extracted from TCGA database, hierarchical clustering was used to detect the sample group data (Fig. 2A), a total of 2197 differential genes were screened out by "limma" R package, and 259 FRGs were extracted from FerrDb. The intersection of the above differential genes was taken, and a Venn

diagram was used for visualization (Fig. 2B). A total of 65 DEFRGs were obtained, and these up- and downregulated genes are presented in a volcano plot (Fig. 2C).

Construction of the ferroptosis-related prognostic model:

Univariate Cox regression analysis was applied to evaluate the potential prognostic value of the above DEFRGs. As a result, four DEFRGs related to the overall survival of CHOL, namely, STMN1, ALOX5, EGFR and BNIP3, were selected for the construction of the prognostic model (Fig. 2D), and multivariate Cox regression analysis was used to calculate the correlation coefficient (Fig. 2E). The riskScore formula is as follows: $\text{RiskScore} = e^{(0.009000742 \times \text{expression of STMN1}) + (-0.137555962 \times \text{expression of EGFR}) + (-0.023056411 \times \text{expression of ALOX5}) + (0.191038641 \times \text{expression of BNIP3}) - 0.09}$. In addition, the patients were divided into a high-risk group (n = 18) and a low-risk group (n = 18) according to the median riskScore (Fig. 3A). As shown in the figure survStat (Fig. 3B), patients in the high-risk group had a significantly shorter survival time, and Kaplan–Meier survival analysis further confirmed our conclusion (P < 0.001, Fig. 3C). In addition, the ROC curve indicated that the riskScore model had higher diagnostic value (AUC = 0.991, Fig. 3D).

To further explore whether the riskScore can become an independent prognostic factor, we performed univariate and multivariate Cox regression analyses, and in the univariate Cox regression analysis, the riskScore was significantly correlated with survival time (HR = 1.302, 95% CI = 1.129-1.501, P < 0.001, Fig. 4A). After removing confounding factors, the riskScore remained a highly valuable independent prognostic factor (HR = 1.312, 95% CI = 1.093-1.573, P = 0.003, Fig. 4B).

Validation of the ferroptosis-related prognostic model:

First, we established a nomogram by combining the clinical data (sex and stage) of patients and the riskScore to further predict the 1-, 3-, and 5-year overall survival of CHOL patients (Fig. 4C).

In addition, of the 45 samples in the TCGA database, STMN1 and ALOX5 were significantly upregulated compared with normal tissues (all P < 0.05), but the expression of BNIP3 was significantly downregulated compared with normal tissues, and there was no significant difference in the expression of EGFR between CHOL and adjacent normal tissues (P < 0.05, Fig. 5A). Besides, to increase the reliability of genes in the model, we downloaded data on 169 cases of CHOL and adjacent sample data containing gene expression from the GSE26566 cohort as the validation set, and unlike the results obtained from TCGA, EGFR was highly expressed in normal samples (P < 0.05, Fig. 5B). In addition, we examined the mRNA expression levels corresponding to the four genes between CHOL tissues and adjacent normal tissues by qRT–PCR (n = 10, P < 0.05, Fig. 5C) and found that the results were consistent with those in TCGA and GEO, except that the expression level of EGFR was not significantly different between cancer and adjacent tissues. Therefore, our findings suggest that STMN1, ALOX5 and BNIP3 may be key genes in this prognostic model. All of the above statistical methods were applied to t-tests, and P < 0.05 was considered statistically significant.

Finally, the survival curves of the above genes were plotted using the Gene Expression Profiling Interactive Analysis (GEPIA) database; patients with low expression of STMN1 and BNIP3 had a long survival time, and those with high expression of ALOX5 versus EGFR had a long survival time (Fig. 5D-G).

Functional enrichment analysis:

First, we performed functional enrichment analysis of 65 DEFRGs to explore the pathways and biological functions involved in CHOL. The GO results showed that these DEFRGs were mainly related to the response to oxidative stress (Fig. 6A). KEGG analysis indicated that these DEFRGs were mainly enriched in ferroptosis, glutathione metabolism, 2-oxocarboxylic acid metabolism and central carbon metabolism in cancer (Fig. 6B).

Correlation between immune infiltration and prognosis of CHOL:

To explore the infiltration of immune cells in CHOL, we analyzed 45 CHOL and paracancerous samples in TCGA, which were divided into a high invasion group and a low invasion group according to the cluster analysis of the degree of immune cell infiltration, and the immunoprofile of CHOL was visualized using ssGSEA (Fig. 7A). Based on this analysis, we found that there were significant differences in the infiltration of immune cells in CHOL. Next, the association between the four DEFRGs and the immune infiltrate microenvironment was further analyzed (Fig. 7B). The results revealed that ALOX5 had the strongest correlations with aDCs ($r = 0.60$, $P < 0.001$) and Th17 cells ($r = -0.45$, $P < 0.01$), BNIP3 was positively correlated with neutrophils ($r = 0.57$, $P < 0.001$) and negatively correlated with NK CD56 dim cells ($r = -0.58$, $P < 0.001$), and STMN1 had a positive correlation with Th2 cells ($r = 0.74$, $P < 0.001$). ALOX5 was negatively correlated with Th17 cells ($r = -0.56$, $P < 0.001$), whereas there was no significant correlation between EGFR and immune cells. Tgd, cytotoxic cells and neutrophils were more significantly enriched in the high-risk group of the prognostic model.

Next, we validated the correlation between immune cells and the prognostic model by immune cell markers, which showed that BNIP3 was negatively correlated with SPON2 and IL-21R, markers of NK CD56dim cells, and strongly positively correlated with CYP4F3 and VNN3, markers of neutrophils (Fig. 8A). ALOX5 was positively correlated with the molecular markers LAMP3 and OAS3 of aDCs and negatively correlated with the immune marker RORC of Th17 cells (Fig. 8B). STMN1 was positively correlated with the biomarkers BIRC5, CDC25C, HELLS, NEIL3 and CENPF of Th2 cells (Fig. 8C), which is consistent with our previous conclusion.

In addition, we explored the pathways related to CHOL by GSEA. The riskScore was positively correlated with tyrosine metabolism, fatty acid metabolism and cytochrome P450 drug metabolism but negatively correlated with complexity and coordination cascades. Enrichment analysis of immune function in CHOL showed that the riskScore was negatively correlated with the IFN- γ signature (Figure. 8D).

Discussion

Cell death has long been considered a typical feature of malignant tumors, as it is very important for maintaining body balance and preventing excessive tumor proliferation. Cancerous cells always show high metabolic levels, antioxidant modification and increased iron intake to maintain unlimited proliferation [16]. The proposal of ferroptosis breaks people's understanding of cell death and emphasizes the important role of excessive iron metabolism in carcinogenesis [17]. In recent years, this unique cell death mode has become a new research hotspot. A considerable number of scholars have explored the mechanism of ferroptosis in tumors, such as hepatocellular carcinoma [18], glioma [19], and lung adenocarcinoma [20]. However, the mechanism of ferroptosis in CHOL is not clear. In our study, we found that these DEFRGs were mainly related to oxidative stress, ferroptosis and tumor metabolism. In addition, a nomogram based on clinicopathological parameters and riskScore was constructed to help clinicians make better decisions and treatment plans. The ROC curve indicated that the model also has high specificity and sensitivity and can be used for the early diagnosis of CHOL. In addition, single-sample immune cell enrichment analysis showed that ferroptosis was closely related to immunity in CHOL. These results suggest that the model has great research value in CHOL.

The GO and KEGG results suggested that the DEFRGs were significantly enriched in ferroptosis, reactive oxygen species (ROS), cell oxidative stress, iron ion reaction, carbon metabolism and other related pathways. These biological processes are crucial for the implementation of ferroptosis [21, 22]. A large number of studies have shown that ferroptosis is a ROS- and iron-dependent cell death mode [23]. Therefore, the close relationship between ferroptosis and oxidative stress can be understood as iron-dependent lipid peroxidation accumulation. Iron chelators and fat-soluble antioxidants can inhibit ferroptosis [24]. We further analyzed the gene enrichment pathway in CHOL by GSEA, which showed that it was significantly enriched in amino acid, fatty acid and other metabolic pathways, and the riskScore was positively correlated with most pathways (Fig. 8D). At present, although great progress has been made in the treatment of CHOL, its prognosis is still poor due to its complex pathogenesis. Therefore, understanding the specific pathogenesis of CHOL is very important for the development of targeted therapy.

It is worth noting that the prognostic model we constructed is closely related to the ferroptosis of tumors. The mitotic receptor BNIP3 is a key mitochondrial autophagy receptor in cells and is necessary to prevent increases in ROS levels and cell death. It is regulated by upstream hypoxia inducible factor (HIF-1) and is highly expressed in normal tissues; it can inhibit the growth of many types of tumors [25, 26]. However, when cells become cancerous, the interaction between inflammation and tumors increases the production of ROS in tumor cells [27,28], and the production of ROS may also be carcinogenic, leading to oxidative DNA damage and genetic instability [29]. At this time, the BNIP3 gene is a risk factor in tumors, which may be the result of the joint action of many factors. The lipase family encoded by the ALOX5 gene plays an important role in leukotriene biosynthesis and participates in a variety of processes, such as inflammation and tumors [28]. In the study of stroke, some scholars proposed that N-acetylcysteine (NAC) can prevent ferroptosis by neutralizing the toxic lipids produced by ALOX5[30]. However, the role of ALOX5 in CHOL needs to be further explored. STMN1 is the main regulator of microtubule dynamics and plays an important role in the regulation of the cell cycle [31], which is closely related to the proliferation

and division of tumor cells [32]. Our experimental results and data in TCGA suggest that EGFR gene expression is not obviously different between CHOL and normal tissues, which may be due to the relatively small sample size; therefore, further experimental studies are necessary to clarify its mechanism.

In recent years, there has been a close relationship between immunity and tumor progression, and it is also a research hotspot. A large number of studies have shown that the tumor immune microenvironment has important value in predicting tumor prognosis and evaluating therapeutic effects [33]. Different types of immune cell infiltration may create a favorable or unfavorable environment for tumor cell proliferation and metastasis [34]. Interestingly, this study found a correlation between the expression level of genes in the prognostic model and immune infiltrating cells.

Among them, BNIP3 has a strong correlation with most immune cells, especially NK CD56dim cells and neutrophils. First, for NK CD56dim cells, increasing evidence supports that NK CD56dim cells play a cytotoxic role mainly by mediating the antitumor response [35, 36]. We also found that the riskScore was negatively correlated with IFN- γ in CHOL (Fig. 8D). Notably, NK CD56dim cells secrete IFN- γ to activate antitumor adaptive immunity in head and neck cancer [37], further confirming our conclusion. Second, it is well known that the presence of neutrophils in tumors is usually related to the poor prognosis of patients because they participate in the proliferation, invasion and immunosuppression of various tumors [38, 39] and induce blood vessels by releasing ROS, reactive nitrogen species (RNS) or protease to promote tumorigenesis [40], which is consistent with the aforementioned poor prognosis of patients with high expression of BNIP3 in CHOL (Fig. 5D).

In addition, we found that the expression of ALOX5 was positively correlated with aDC and its molecular markers LAMP3 and OAS3. From the cluster analysis of immune cells (Fig. 6C), aDCs were positively correlated with NK CD56dim cells. Therefore, aDCs may also play an antitumor role in tumors. LAMP3 is induced by hypoxia and is upregulated in esophageal cancer [41]. Another molecular marker, OAS3, is an important part of interferon-induced antiviral enzymes, and studies have shown that the high expression of OAS3 in breast cancer is closely related to prognosis [42]. The expression of ALOX5 is negatively correlated with Th17 cells. Some studies have shown that in colorectal cancer, patients with high infiltration of Th17 and high expression of its immune marker RORC have a poor prognosis [43]. This seems to be one of the reasons why ALOX5 is highly expressed in CHOL, but the prognosis of patients is better (Fig. 5E).

The oncogene STMN1 is positively correlated with Th2 cells in CHOL. Studies have shown that patients with high tumor malignancy have a low Th1/Th2 ratio; generally speaking, tumor cells produce immunosuppressive cytokines and prostaglandins, which relatively enhance the immune effect of Th2 cells and significantly reduce the antitumor ability [43,44,45]. Interestingly, we know that BIRC5, CDC25C, HELLS, NEIL3 and CENPF are common immune markers of Th2 cells. Some scholars believe that BIRC5 plays an important role in cell division and can inhibit apoptosis [46]. In hepatocellular carcinoma, the upregulation of BIRC5 expression promotes the proliferation of tumor cells [47]. CDC25C plays a

significant role in regulating the cell cycle and is highly expressed in prostate cancer [48]. The high expression of HELLS and NEIL3 is related to the shortening of survival time associated with liver cancer and other tumors [49, 50]. The upregulated expression of CENPF is closely related to the poor prognosis and progression of liver cancer, lung adenocarcinoma and other tumors [51, 52]. These findings seem to explain the poor prognosis of patients with high expression of STMN1 in CHOL (Fig. 5F). In addition, patients with high EGFR expression had a better prognosis than patients with low EGFR expression (Fig. 5G).

Our study has some limitations. First, the amount of patient information available in public databases is insufficient, and clinical staging, treatment options and other valuable data could not be obtained. In addition, our experimental validation results are slightly different from those in the public database, possibly due to the insufficient sample sizes or differences in ethnic origin; therefore, a large amount of clinicopathological information needs to be collected later for prospective studies. Moreover, the mechanism by which the DEFRGs regulated CHOL in this prognostic model needs to be explored in further experimental studies.

Conclusions

In conclusion, this study is the first to construct a polygenic prognostic model of ferroptosis in CHOL, showing that ALOX5, BNIP3, EGFR and STMN1 act together to strongly contribute to CHOL cell apoptosis, ferroptosis and immune microenvironment, and also confirmed the diagnostic and prognostic value of this model in CHOL. As relevant studies are scarce, this topic also provides ideas and directions for research related to ferroptosis in CHOL and provides a reference for finding new treatment modalities.

Declarations

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Authors' contributions

ZW designed the study, analyzed the data and drafted the manuscript. XC and ZW performed the data analysis. ZW and ZJ revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The data used in this study are obtained from TCGA (<https://portal.gdc.cancer.gov/>) and GEO (<https://portal.gdc.cancer.gov/>).

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Written informed consent for this study was provided by patients / participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Banales JM, Cardinale V, Carpino G, Marzioni M, Andersen JB, Invernizzi P, Lind GE, Folseraas T, Forbes SJ, Fouassier L, Geier A, Calvisi DF, Mertens JC, Trauner M, Benedetti A, Maroni L, Vaquero J, Macias RI, Raggi C, Perugorria MJ, Gaudio E, Boberg KM, Marin JJ, Alvaro D. Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA). *Nat Rev Gastroenterol Hepatol*. 2016 May;13(5):261-80.
2. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. *Gastroenterology*. 2013 Dec;145(6):1215-29.
3. Valle JW, Borbath I, Khan SA, Huguet F, Gruenberger T, Arnold D; ESMO Guidelines Committee. Biliary cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2016 Sep;27(suppl 5): v28-v37.
4. Lendvai G, Szekerczés T, Illyés I, Dóra R, Kontsek E, Gógl A, Kiss A, Werling K, Kovalszky I, Schaff Z, Borka K. Cholangiocarcinoma: Classification, Histopathology and Molecular Carcinogenesis. *Pathol Oncol Res*. 2020 Jan;26(1):3-15.
5. Rodrigues PM, Olaizola P, Paiva NA, Olaizola I, Agirre-Lizaso A, Landa A, Bujanda L, Perugorria MJ, Banales JM. Pathogenesis of Cholangiocarcinoma. *Annu Rev Pathol*. 2021 Jan 24; 16: 433-463.

6. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, Morrison B 3rd, Stockwell BR. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*. 2012 May 25;149(5):1060-72.
7. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, Fulda S, Gascón S, Hatzios SK, Kagan VE, Noel K, Jiang X, Linkermann A, Murphy ME, Overholtzer M, Oyagi A, Pagnussat GC, Park J, Ran Q, Rosenfeld CS, Salnikow K, Tang D, Torti FM, Torti SV, Toyokuni S, Woerpel KA, Zhang DD. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell*. 2017 Oct 5;171(2):273-285.
8. Li P, Jiang M, Li K, Li H, Zhou Y, Xiao X, Xu Y, Krishfield S, Lipsky PE, Tsokos GC, Zhang X. Glutathione peroxidase 4-regulated neutrophil ferroptosis induces systemic autoimmunity. *Nat Immunol*. 2021 Sep;22(9):1107-1117.
9. Xu M, Tao J, Yang Y, Tan S, Liu H, Jiang J, Zheng F, Wu B. Ferroptosis involves in intestinal epithelial cell death in ulcerative colitis. *Cell Death Dis*. 2020 Feb 3;11(2):86.
10. Liang JY, Wang DS, Lin HC, Chen XX, Yang H, Zheng Y, Li YH. A Novel Ferroptosis-related Gene Signature for Overall Survival Prediction in Patients with Hepatocellular Carcinoma. *Int J Biol Sci*. 2020 Jul 6;16(13):2430-2441.
11. Jiang X, Yan Q, Xie L, Xu S, Jiang K, Huang J, Wen Y, Yan Y, Zheng J, Tang S, Nie K, Zheng Z, Pan J, Liu P, Huang Y, Yan X, Zou Y, Chen X, Liu F, Li P, Zhuang K. Construction and Validation of a Ferroptosis-Related Prognostic Model for Gastric Cancer. *J Oncol*. 2021 Feb 28;2021:6635526.
12. Wohlhieter CA, Richards AL, Uddin F, Hulton CH, Quintanal-Villalonga À, Martin A, de Stanchina E, Bhanot U, Asher M, Shah NS, Hayatt O, Buonocore DJ, Rekhtman N, Shen R, Arbour KC, Donoghue M, Poirier JT, Sen T, Rudin CM. Concurrent Mutations in STK11 and KEAP1 Promote Ferroptosis Protection and SCD1 Dependence in Lung Cancer. *Cell Rep*. 2020 Dec 1;33(9):108444.
13. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pagès F, Speicher MR, Trajanoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity*. 2013 Oct 17;39(4):782-95.
14. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, Schinzel AC, Sandy P, Meylan E, Scholl C, Fröhling S, Chan EM, Sos ML, Michel K, Mermel C, Silver SJ, Weir BA, Reiling JH, Sheng Q, Gupta PB, Wadlow RC, Le H, Hoersch S, Wittner BS, Ramaswamy S, Livingston DM, Sabatini DM, Meyerson M, Thomas RK, Lander ES, Mesirov JP, Root DE, Gilliland DG, Jacks T, Hahn WC. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature*. 2009 Nov 5;462(7269):108-12.
15. Chen X, Jiang Z, Wang Z, Jiang Z. The prognostic and immunological effects of ZBTB7C across cancers: friend or foe? *Aging (Albany NY)*. 2021 May 4;13(9):12849-12864. doi: 10.18632/aging.202955. Epub 2021 May 4. PMID: 33946045; PMCID: PMC8148469. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, Fulda S, Gascón S, Hatzios SK, Kagan VE, Noel K, Jiang X, Linkermann A, Murphy ME, Overholtzer M, Oyagi A, Pagnussat GC, Park J, Ran Q,

- Rosenfeld CS, Salnikow K, Tang D, Torti FM, Torti SV, Toyokuni S, Woerpel KA, Zhang DD. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell*. 2017 Oct 5;171(2):273-285.
16. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, Fulda S, Gascón S, Hatzios SK, Kagan VE, Noel K, Jiang X, Linkermann A, Murphy ME, Overholtzer M, Oyagi A, Pagnussat GC, Park J, Ran Q, Rosenfeld CS, Salnikow K, Tang D, Torti FM, Torti SV, Toyokuni S, Woerpel KA, Zhang DD. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell*. 2017 Oct 5;171(2):273-285.
17. Toyokuni S, Ito F, Yamashita K, Okazaki Y, Akatsuka S. Iron and thiol redox signaling in cancer: An exquisite balance to escape ferroptosis. *Free Radic Biol Med*. 2017 Jul;108:610-626.
18. Liu Y, Zhang X, Zhang J, Tan J, Li J, Song Z. Development and Validation of a Combined Ferroptosis and Immune Prognostic Classifier for Hepatocellular Carcinoma. *Front Cell Dev Biol*. 2020 Dec 23;8:596679.
19. Zhuo S, Chen Z, Yang Y, Zhang J, Tang J, Yang K. Clinical and Biological Significances of a Ferroptosis-Related Gene Signature in Glioma. *Front Oncol*. 2020 Nov 20;10:590861.
20. Zhang A, Yang J, Ma C, Li F, Luo H. Development and Validation of a Robust Ferroptosis-Related Prognostic Signature in Lung Adenocarcinoma. *Front Cell Dev Biol*. 2021 Jun 24;9:616271.
21. Hassannia B, Vandenabeele P, Vanden Berghe T. Targeting Ferroptosis to Iron Out Cancer. *Cancer Cell*. 2019 Jun 10;35(6):830-849.
22. Lelièvre P, Sancey L, Coll JL, Deniaud A, Busser B. Iron Dysregulation in Human Cancer: Altered Metabolism, Biomarkers for Diagnosis, Prognosis, Monitoring and Rationale for Therapy. *Cancers (Basel)*. 2020 Nov 26;12(12):3524.
23. Xie Y, Hou W, Song X, Yu Y, Huang J, Sun X, Kang R, Tang D. Ferroptosis: process and function. *Cell Death Differ*. 2016 Mar;23(3):369-79.
24. Dixon SJ, Winter GE, Musavi LS, Lee ED, Snijder B, Rebsamen M, Superti-Furga G, Stockwell BR. Human Haploid Cell Genetics Reveals Roles for Lipid Metabolism Genes in Nonapoptotic Cell Death. *ACS Chem Biol*. 2015 Jul 17;10(7):1604-9.
25. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ, Semenza GL. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem*. 2008 Apr 18;283(16):10892-903.
26. Li L, Tan J, Miao Y, Lei P, Zhang Q. ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. *Cell Mol Neurobiol*. 2015 Jul;35(5):615-21.
27. Prevete N, Liotti F, Amoresano A, Pucci P, de Paulis A, Melillo RM. New perspectives in cancer: Modulation of lipid metabolism and inflammation resolution. *Pharmacol Res*. 2018 Feb;128:80-87.
28. Chourasia AH, Macleod KF. Tumor suppressor functions of BNIP3 and mitophagy. *Autophagy*. 2015;11(10):1937-8. doi: 10.1080/15548627.2015.1085136.
29. Güngör N, Knaapen AM, Munnia A, Peluso M, Haenen GR, Chiu RK, Godschalk RW, van Schooten FJ. Genotoxic effects of neutrophils and hypochlorous acid. *Mutagenesis*. 2010 Mar;25(2):149-54.

30. Karuppagounder SS, Alin L, Chen Y, Brand D, Bourassa MW, Dietrich K, Wilkinson CM, Nadeau CA, Kumar A, Perry S, Pinto JT, Darley-Usmar V, Sanchez S, Milne GL, Pratico D, Holman TR, Carmichael ST, Coppola G, Colbourne F, Ratan RR. N-acetylcysteine targets 5 lipoxygenase-derived, toxic lipids and can synergize with prostaglandin E2 to inhibit ferroptosis and improve outcomes following hemorrhagic stroke in mice. *Ann Neurol*. 2018 Dec;84(6):854-872.
31. Steinmetz MO. Structure and thermodynamics of the tubulin-stathmin interaction. *J Struct Biol*. 2007 May;158(2):137-47.
32. Belletti B, Baldassarre G. Stathmin: a protein with many tasks. New biomarker and potential target in cancer. *Expert Opin Ther Targets*. 2011 Nov;15(11):1249-66.
33. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, Coussens LM, Gabrilovich DI, Ostrand-Rosenberg S, Hedrick CC, Vonderheide RH, Pittet MJ, Jain RK, Zou W, Howcroft TK, Woodhouse EC, Weinberg RA, Krummel MF. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med*. 2018 May;24(5):541-550.
34. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol*. 2013 Oct;14(10):1014-22.
35. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol*. 2001 Nov;22(11):633-40.
36. Jonges LE, Albertsson P, van Vlierberghe RL, Ensink NG, Johansson BR, van de Velde CJ, Fleuren GJ, Nannmark U, Kuppen PJ. The phenotypic heterogeneity of human natural killer cells: presence of at least 48 different subsets in the peripheral blood. *Scand J Immunol*. 2001 Feb;53(2):103-10.
37. Mandal R, Şenbabaoğlu Y, Desrichard A, Havel JJ, Dalin MG, Riaz N, Lee KW, Ganly I, Hakimi AA, Chan TA, Morris LG. The head and neck cancer immune landscape and its immunotherapeutic implications. *JCI Insight*. 2016 Oct 20;1(17):e89829.
38. Swierczak A, Mouchemore KA, Hamilton JA, Anderson RL. Neutrophils: important contributors to tumor progression and metastasis. *Cancer Metastasis Rev*. 2015 Dec;34(4):735-51.
39. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer*. 2016 Jul;16(7):431-46.
40. Antonio N, Bønnelykke-Behrndtz ML, Ward LC, Collin J, Christensen IJ, Steiniche T, Schmidt H, Feng Y, Martin P. The wound inflammatory response exacerbates growth of pre-neoplastic cells and progression to cancer. *EMBO J*. 2015 Sep 2;34(17):2219-36.
41. Liao X, Chen Y, Liu D, Li F, Li X, Jia W. High Expression of LAMP3 Is a Novel Biomarker of Poor Prognosis in Patients with Esophageal Squamous Cell Carcinoma. *Int J Mol Sci*. 2015 Jul 31;16(8):17655-67.
42. Zhang Y, Yu C. Prognostic characterization of OAS1/OAS2/OAS3/OASL in breast cancer. *BMC Cancer*. 2020 Jun 19;20(1):575.
43. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman WH, Pagès F, Galon J. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res*. 2011 Feb 15;71(4):1263-71.

44. Loose D, Van de Wiele C. The immune system and cancer. *Cancer Biother Radiopharm.* 2009 Jun;24(3):369-76.
45. Kumar S, Saini RV, Mahindroo N. Recent advances in cancer immunology and immunology-based anticancer therapies. *Biomed Pharmacother.* 2017 Dec;96:1491-1500.
46. Wheatley SP, Altieri DC. Survivin at a glance. *J Cell Sci.* 2019 Apr 4;132(7):jcs223826.
47. Cao L, Li C, Shen S, Yan Y, Ji W, Wang J, Qian H, Jiang X, Li Z, Wu M, Zhang Y, Su C. OCT4 increases BIRC5 and CCND1 expression and promotes cancer progression in hepatocellular carcinoma. *BMC Cancer.* 2013 Feb 22;13:82.
48. Ozen M, Ittmann M. Increased expression and activity of CDC25C phosphatase and an alternatively spliced variant in prostate cancer. *Clin Cancer Res.* 2005 Jul 1;11(13):4701-6.
49. Law CT, Wei L, Tsang FH, Chan CY, Xu IM, Lai RK, Ho DW, Lee JM, Wong CC, Ng IO, Wong CM. HELLS Regulates Chromatin Remodeling and Epigenetic Silencing of Multiple Tumor Suppressor Genes in Human Hepatocellular Carcinoma. *Hepatology.* 2019 May;69(5):2013-2030.
50. Wang W, Yin Q, Guo S, Wang J. NEIL3 contributes toward the carcinogenesis of liver cancer and regulates PI3K/Akt/mTOR signaling. *Exp Ther Med.* 2021 Oct;22(4):1053.
51. Si T, Huang Z, Jiang Y, Walker-Jacobs A, Gill S, Hegarty R, Hamza M, Khorsandi SE, Jassem W, Heaton N, Ma Y. Expression Levels of Three Key Genes CCNB1, CDC20, and CENPF in HCC Are Associated With Antitumor Immunity. *Front Oncol.* 2021 Sep 30;11:738841.
52. Li MX, Zhang MY, Dong HH, Li AJ, Teng HF, Liu AL, Xu N, Qu YQ. Overexpression of CENPF is associated with progression and poor prognosis of lung adenocarcinoma. *Int J Med Sci.* 2021 Jan 1;18(2):494-504.

Figures

Figure 1

Flow chart of our study.

Figure 2

A The use of the hierarchical clustering method for the classification of 9 normal and 36 tumor samples. **B** The intersection of 2197 differential genes and 259 FRGs was visualized by Venn diagram, and 65 DEFRGs were ultimately screened. **C** Volcano plot of all genes. **D** Exploration of the prognosis of DEFRGs. The results of the univariate Cox regression analysis of overall survival in CHOL. **E** The coefficients of 4 DEFRGs

Figure 3

Validation of the riskScore prognostic model in TCGA-CHOL cohort. **A** Distribution of riskScore, green dots indicate the patients in the low-risk group, red dots indicate high-risk group. **B** Survival status of high- and low-risk patients, red dots represent the dead patients, the green dots represent people alive. **C** Kaplan-Meier survival curve for OS. **D** The AUC of ROC curve evaluated the diagnostic value of the riskScore in the CHOL cohort.

Figure 4

A-B Univariate **(A)** and multivariate **(B)** Cox regression analyses to evaluate the relationship between the riskScore and clinical data and OS in CHOL cohort. **C** Establishing a nomogram based on the signature to predict the 1-, 3-, 5-year OS in CHOL patients.

Figure 5

A-C The differential expression level of mRNA of the four DEFRGs in CHOL and normal tissues. **(A)** Data from the TCGA. **(B)** Data from the GEO. **(C)** 10 pairs of fresh CHOL specimens and adjacent normal tissue specimens from the First Affiliated Hospital of Chongqing Medical University. **D-G** The overall survival curve of CHOL patients based on the expression of BNIP3, ALOX5, STMN1 and EGFR.

Figure 6

A-B Functional enrichment analysis of the 65 DEFRGs in CHOL, showing the top 10 enriched BP, CC and MF (A), and KEGG pathways (B).

A

B

C

Figure 7

A The 45 patients from TCGA-CHOL cohort were clustered using the single-sample gene set enrichment analysis scores from 24 immune cell types, the mutation status of ALOX5, STMN1, BNIP3 and EGFR, gender, survival as well as stage were showed in the lower panel. High infiltration and low infiltration were defined by hierarchical clustering analysis. **B** Correlations between the DEFRGs, riskScore and 24 immune

cells, blue boxes indicate negative correlation, red boxes indicate positive correlation. **C** The cluster analysis of 24 immune cells, which were divided into four categories.

Figure 8

A-C Correlations between BNIP3, ALOX5, STMN1 and immune biomarkers in the TCGA-CHOL cohort. **D** Correlations between riskScore and immunotherapy-predicted pathways (left half of Fig. 8D) and correlations between riskScore and enriched pathways of the gene sets in CHOL (right half of Fig. 8D). BP, biological process; CC, cellular component; MF, molecular function.